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STUDIES ON ARBORVIRUSES AND THEIR EXTRACTS BY MEANS OF CHROMATOGRAPHY ON ION-EXCHANGE CELLULOSES AND GEL-FILTRATION ON AGAROSE*

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Cheng's work [9] has shown that, in the case of the virus from the Semliki forest, the hemagglutinating activity of the arborvirus is associated with the supporting virions. While this work was in progress Mussgay and Rott [24] have demonstrated the same thing for the Sindbis virus. It also seemed possible to separate, by chromatography, a soluble hemagglutinin bound to the virus (Smith and Holt [36], and Gajdamovic and Doan [13]).

The presence, in the coat of the virus, of lipids which come from the host cell (Pfefferkorn and Hunter [29]) explains the well-known sensitivity of arborviruses toward sodium desoxycholate and ether.

This investigation deals with:

1. Search for the presence of a soluble hemagglutinin by means of new chromatographic procedures.

2. Application of separation methods which are based on the size of the particles.

3. Investigation of the products formed on treatment with ether, alone or with ether plus a detergent.

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Material and Techniques

Material. Group A virus strains: Sindbis AR 339 (26-27th transfer), Chikungunya S 27 (176-177th transfer). Group B: West Nile B 956 (42-45th transfer), Zika MR 776 (23-24th transfer). Group D: Bunyamwera MG 967 (17-18th transfer). Strains from young mouse brain. The mice were inoculated, the day they were born, by intracerebral route.

Titration of the infectious power. These titrations are carried out either on newborn mice (effluents of chromatography on cellulose) according to a method based on that of Riley /34/, or by counting of plaques (Sindbis, Chikungunya, Bunyamwera viruses) on a chicken fibroblast culture according to a technique derived from that of Porterfield /32/ with the following modifications: cell growth in a casein hydrolyzate medium containing 10% calf serum /22, 15/ in Petri dishes in a 90:10 air-CO_2 atmosphere; density of the cell inoculum: 2.10^5 cells/cm^2 of glass surface, this yields a continuous layer in 24 hours; volume of the viral inoculum: 0.5 ml for 50 cm^2; adsorption time: 30 minutes; covered by a mixture of equal volumes of double casein hydrolyzate and 10% serum of newborn, precolostral calf (Colorado Serum Company)-gelose Noble (Difco); height of layer of medium: 0.2 cm; development of the plaques, after 24 hours, by adding 1 ml 0.2% neutral red to the surface of the medium; reading taken on the second day (Sindbis) and on the third day (Chikungunya, Bunyamwera).

Extraction of the Hemagglutinins. Extraction starting with brain suspensions or culture media: in a saccharose medium with acetone at -30° C /10/. For the Sindbis virus hemagglutinins are also obtained from chicken fibroblast cultures: The cell layers, prepared as described above, are washed twice with a large volume of Hanks solution, inoculated with 0.5 ml of virus dilution and, after adsorption for 30 minutes, covered, without washing, with 10 ml of casein hydrolyzate medium without serum (some lots of newborn, precolostral calf serum contain inhibitors). Harvesting of a unit of plaque formation per cell: after 24 hours for a multiple infection; after 48-84 hours for lower multiple infections. In the same fashion the Bunyamwera hemagglutinins are obtained on EpO cells from mouse ependymoma /28/.

Titration of the hemagglutinating and inhibiting power of the immune sera is carried out according to /10/. Production of immune sera in guinea pigs and in rabbits according to the procedures of /32/.

Chromatography on Anion Exchange Cellulose. Adsorbents: Anion exchange celluloses: DEAE cellulose, 0.90 meq/g; ECTEOLA cellulose, 0.35 meq/g; cation exchangers: CM cellulose, 0.50 meq/g (Serva).

Dimensions of columns usually 10 x 1.6 cm; rate of flow 20-25 ml/hr; elution by a series of 0.01 M phosphate buffers, pH 8, with increasing concentration of NaCl or by linear concentration gradient at constant pH. During the entire chromatography the temperature is maintained between +2 and +4° C; this condition is essential to avoid the denaturing of the virus

on the column. Chromatography of 10% viral suspensions in 0.05 M borate buffer, 0.12 M NaCl, pH 9, to which 0.4% beef albumin has been added, or by preparations extracted with acetone. The effluents are read at 2,600 and 2,800 Å.

Control and titer of the hemagglutinating fractions, and eventually control of the infectious and inhibitory power of the fractions, were also carried out.

Gel-Filtration. On a slightly cross-linked dextran (Sephadex, AB Pharmacia, Uppsala) and on agarose gel granules (Industrie Biologique Francaise). The theory and applications of this method have been reviewed recently /11/.

The first viruses to be separated on gelose gel were plant viruses /38/.

Agarose was used according to Hjerten /16/ because it does not have polar groups like agaropectin: the second poly-ose constituent of gelose; high molecular weight dextran sulfate (biological equilibrium) has -- in the presence of Ca^{++} -- a precipitating effect on arborvirus (more than 75% of the hemagglutinating activity of the virus (Sindbis and West-Nile) are entrained by the precipitate). This is analogous to the case of heparin (or synthetic heparinoids) and herpes virus /25/. Recently Schultze /35/ has also demonstrated the inhibition of the dengue virus by agaropectin.

The gel granules are usually prepared according to the procedure of Bengtsonn and Philipson /5/. Agarose is dissolved, by heating, in isotonic borate buffer, pH 9. Concentration varies between 2 and 3%. The agarose solution is dispersed, in 100-200 ml fractions at 85° C, in about 400 ml ethyl ether which is initially at -5° C. The preparation is agitated vigorously during the process. The respective temperatures and volumes of ether and agarose are critical. The ether is decanted and the agarose granules are washed several times with distilled water so as to remove the residual ether, finally they are filtered through nylon. The column, under slight excess pressure, is loaded with a thick borate buffer suspension of agarose granules. The dimensions of the column vary between 30 x 1 cm and 50 x 0.9 cm. The correct filling of the columns is checked by observing the migration of a band of colored material (usually hemoglobin) added to the gel.

The empty volume (total volume of the column less the volume of the gel granules) is determined by observing /measuring/ the elution volume of a large-size virus (Bunyamwera). For the columns used this volume is 20-30% of the total volume.

To obtain a good separation it seems essential,

1. That the lengths which the particles excluded from the gel have to traverse be as little dispersed as possible, which requires a correct granulation and filling of the columns;

2. That the flow be kept at such a rate that the transverse diffusion of the non-excluded substances between gel and empty volume, be complete, and that this phenomenon not be deformed by an excess longitudinal diffusion. In agreement with Andrews /4/, we find that 4 ml/hr is optimal; but rates of 20 ml/hr have been used, rather satisfactorily, for long columns.

Results

1. CHROMATOGRAPHY ON ION EXCHANGE CELLULOSE COLUMNS

a) Chromatography on ECTEOLA cellulose has already been used for the purification of several arboviruses of the B group /39/. Chromatography on DEAE cellulose of a brain suspension of the only investigated B virus (West Nile) permits elution with a salt concentration identical to that of the purified preparation. The yield, however, is poor. This property of the viruses of the B group must be considered in view of the fact that their hemagglutinins can be demonstrated by simple dilution in an alkaline medium. For the other groups neither the virus, nor the substances which have the power to inhibit the hemagglutinin are found in the effluent, which suggests that the virus, bound to the cerebral inhibitor, is irreversibly fixed onto the column.

These difficulties have been resolved to a large extent by extraction of the lipoproteins with acetone at -30° C after checking that the particles in the virus preparation are still of the original size (see Section 2) and are infective.

Table 1. Chromatography on Ion-Exchange Cellulose. Fractional Elution

Exchanger	Yield	Molarity of Fractions	Sindbis (A)	Zika (B)	West-Nile (B)	Bunyamwera (D)
DEAE	Partial*	0,2	0		0	0
		0,3	21		0	0
		0,4	69		100	0
		0,5	10		0	100
	Total*		71		22	34
ECTEOLA	Partial*	0,1	5	0	0	14
		0,2	76	100	52	58
		0,35	10	0	40	21
		0,5	9	0	8	7
	Total*		47	37	75	38

* The partial yields are expressed as percent of the eluted hemagglutinins, the total yield as percent of the hemagglutinin fixed on the column (average of a series of 2 or 3 experiments).

b) Under these conditions the results of the arboviruses, fractionally eluted at various salt concentrations, are summarized in Table I. Very poor yields are obtained from GE-cellulose and this material has not been retained for a more detailed investigation.

Altogether, and not taking the sharper demarcation on ECTEOLA cellulose into account, the viruses are eluted at rather high salt concentrations. They form a homogeneous block which usually has a well-defined front. An example is shown in Figure 1 (Bunyamwera virus).

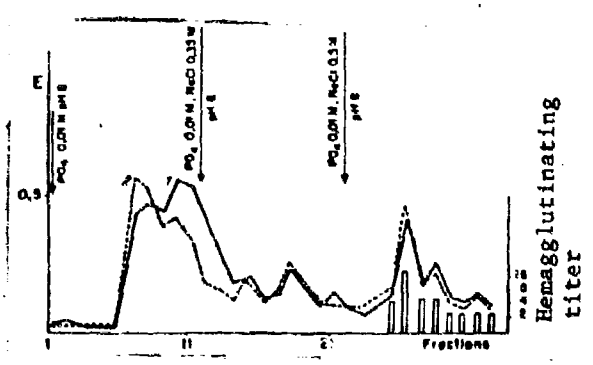

Figure 1. Chromatography of Bunyamwera virus on DEAE cellulose. Adsorbent (0.9 meq) 3 gm; column measuring 10 x 1.6 cm; brain tissue suspension preliminarily extracted with acetone = 1 ml; elution with salt solutions of graded concentrations; 3 ml fractions (E^{1}_{2800}: broken line; E^{1}_{2600}: solid line; hemagglutinating titer = histograms).

The following facts have been verified:

1. The hemagglutinins are eluted at the same time as the infectious power. Especially, the Sindbis virus is eluted, at two different concentration gradients, from DEAE-cellulose, with a fairly constant hemagglutinin infectivity ratio. Inversely the infectious power is not found outside the hemagglutinating fractions.

2. No hemagglutinating fractions, that could be precipitated by protamine sulfate, were found.

3. The hemagglutinating fractions of one virus (Bunyamwera) have been rechromatographed, on CM-cellulose, under the same conditions: they have been eluted as a single peak by graded concentrations of salt solutions.

c) These findings are supplemented by an investigation of the elution behavior of "mixed" virus groups, two viruses per group, which are eluted by linearly increasing concentrations of salt solutions. Identification in the effluent is made by two series of titrations carried out at the

optimum pH of each virus. The initial and final elution molarities are calculated by interpolation. The results are presented in Figure 2 and Table 2. The results agree with those obtained previously (zone 2 of the table) with the exception of the Bunyamwera virus, which is partly eluted at a lower concentration. The only likely explanation is that there is a competition between viruses for the exchange sites. This hypothesis would explain both this phenomenon and the increased yield of the Bunyamwera virus, a fact also supported by the strong adsorption of the West Nile virus.

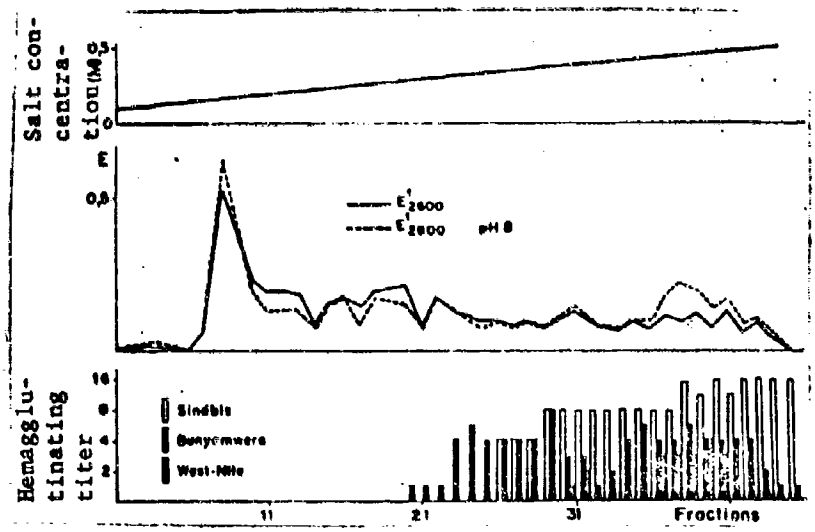

Figure 2. Chromatography of the Bunyamwera (D) West Nile (B) Sindbis (A) virus on DEAE cellulose.
Adsorbent 3 gm, elution by linear gradient. The figure is based on two experiments in which West-Nile-Sindbis and West-Nile-Bunyamwera are associated under the same conditions (0.5 ml of each suspension).

d) The adsorption capacity of the columns could not be determined because of the relatively small amount of material available for each experiment. In the absence of this figure, comparable amounts of virus have been used in each experiment.

Elution of virus at high molarities makes it possible to consider the chromatography as a purification procedure. (Figure 1) As an example the UV spectra of a West Nile preparation at various stages of purification are given in Figure 3. These curves have not been corrected for light diffusion.

Under these conditions the chromatographic behavior could serve as a marker for virus species. Further experiments will have to decide whether they can also serve as intraspecific markers, capable of distinguishing between variants, as is the case for other groups.

Table 2. Chromatography on Ion-Exchange Cellulose. Elution by Linear Gradient

Virus	Zone 1		Zone 2		
	Limits of Elution Zones	Partial Yield*	Limits of Elution Zones	Partial Yield*	Total Yield*
Sindbis (A)	--	—	0,32 - 0,5 M	100	80
West-Nile (B)	0,36 M	(15)	0,41 - 0,44 M	(85)	18
Bunyamwera (B)	0,29 - 0,39 M	42	0,39 - 0,46 M	58	62

* The partial yields are expressed as percent of the total eluted hemagglutinin, the total yield as percent of the fixed hemagglutinin.

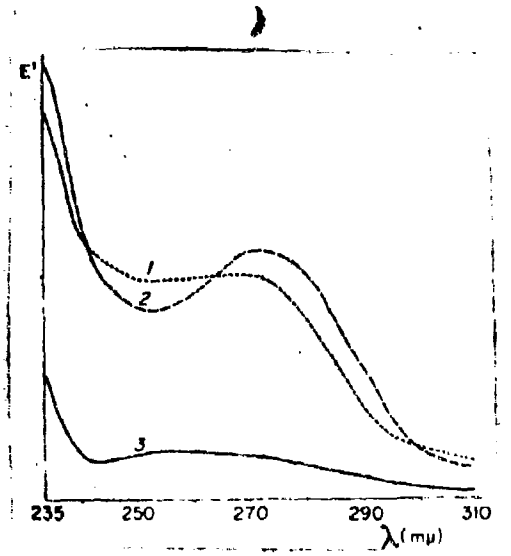

Figure 3. UV spectra of West Nile virus preparation.
1 = brain suspension in borate buffer.
2 = first extraction with cold acetone.
3 = hemagglutinating fraction purified on DEAE cellulose. Curves are not corrected for light diffusion.

2. GEL-FILTRATION OF VIRIONS ON AGAROSE

A dextran gel (Sephadex G 75) has already been used for the purification of the virus of the Murray Valley fever [1]. Dextran gels with a smaller degree of cross linking have been used in this work; the hemagglutinins and the virions are excluded from the gel. The investigation is completed by using material (agarose gel) which separates the particles from the macromolecules.

Table 3. Gel-Filtration on Agarose Columns of Virions and Products Obtained After Treatment With Ether and Detergents.

Virus	Origine (a)	Traitement (b)	Concentration agarose (%)	Nbre d'expériences (c)	Fraction 1		Fraction 2		Rendement total (%)[2] (e)
					Rf[1]	Rendement partiel[2] (d)	Rf[1]	Rendement partiel[2] (d)	
Sindbis	Culture (f) cellulaire[3]	—	2 et 2,5	5	1	100	—	—	95
	Cerveau- (g) acétone	—	2,5	3	1	84	0,78 - 0,50	16	86
	Culture (f) cellulaire[3]	Tween-éther	2 et 2,5	4	—	—	0,93 - 0,54	100	52
	Cerveau- (g) acétone	éther	2,5	2	1	18	0,78 - 0,50	82	
Chikungunya	Cerveau- (g) acétone	—	2,5	2	1	65	0,73 - 0,61	35	87
West-Nile	Cerveau- (g) acétone	—	2,5	1	1	62	0,83 - 0,33	38	
Bunyamwera	Cerveau- (g) acétone	—	1,5-2,5 et 3	3	1	89	0,63	11	100
	Cerveau- (g) acétone	éther	2,5 et 3	2	1	2	0,72 - 0,50	98	

Key: (a) Origin; (b) Treatment; (c) Number of Experiments; (d) Partial Treatment[2]; (e) Total Yield (%); (f) Cell culture[3]; (g) Brain-acetone.

1) Rf = Vo/Ve, where Vo = empty volume and Ve = elution volume.
2) The partial yield is expressed as percent of the eluted hemagglutinins, the total yield as percent hemagglutinin deposited on the column.
3) Virus from cell cultures in the absence of inhibitor.

Preliminary investigation, carried out according to Polson's techniques /30/ indicates that virus particles remained excluded, after 48 hours, from gels with an agarose concentration greater than or equal to 2%.

This is also true for columns of agarose granules used for the gel-filtration of virions preliminarily purified by acetone extraction, at -30° C, of brain suspensions or obtained from tissue cultures. The virions are excluded when the agarose concentration of the gel is equal to or greater than 2% (Table 3). The infectious particles are eluted from the gel with the hemagglutinating fractions in the volume which is excluded from the gel (Figures 4 and 5). However, the acetone-purified virus preparations, obtained from mouse brain, are not homogeneous: a variable amount, depending on the lot, consists of hemagglutinating particles which are not excluded from the gel. The results presented, agree, on the one hand, with the results based on the known size of the arborviruses under investigation (Smithburn and Bugher /37/ and Rott /24/), and on the other hand with a value of 70 for the coefficient K which, according to Polson /30/ establishes the exclusion concentration of agar gels. (C.d = K, where C is the gelose concentration and d is the maximum diameter, in mμ, of the particles admitted to the gel.) The figures agree with the concentrations used by Bengtsonn and Philipson /5/ to separate myxo- and entero-viruses.

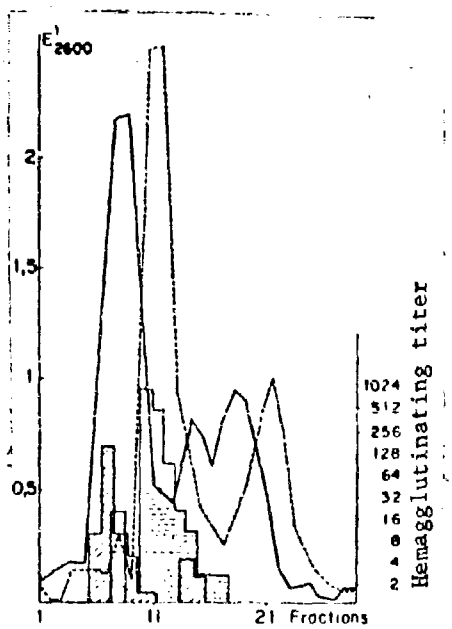

Figure 4. Gel infiltration on agarose of Bunyamwera virus and its products after ether treatment.
Concentration of agarose = 3%. Column 21 x 1 cm. Rate of flow 4 ml/hr. Consecutive gel-filtration of an original brain-acetone virus preparation (solid line) and one treated with ether (dotted line).

Yield is satisfactory and is in agreement with the neutrality of the agarose.

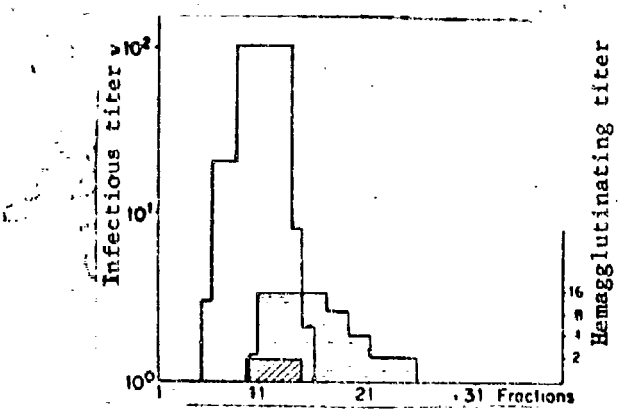

Figure 5. Gel-filtration on agarose of Sindbis virus and its products after treatment with Tween 80 and ether.
Agarose concentration = 2%. Column 53 x 0.9 cm. Rate of flow = 20 ml/hr. Consecutive gel-filtrations of tissue culture virus preparations (infectious titer = white histogram; hemagglutinating titer = cross-hatched histogram); and treated with Tween 80 and ether (hemagglutinating titer histogram indicated by broken lines).

3. ACTION OF DETERGENTS AND ETHER

a) Destruction of the infectiveness of the arborviruses by sodium desoxycholate [39] and ether [7, 14] is a characteristic of the group, which has been verified, and used as an indicator in the present investigation. The effect of phospholipase A, which has been reported in the literature [2] seems to follow the same mechanism.

b) The investigation of the resistance of the hemagglutinin is carried out under the following conditions:

1. Virus in borate buffer, pH 9, sodium desoxycholate 2.5 x 10^{-3} M, one hour at 37° C, formaldehyde treated goose erythoscytes.

2. Phospholipase A from Bitis gabonica venom, from zero to thirty minutes at 37° C; the presence of a venom protease, which has the same specificity as trypsin is enough to account for the partial inactivation of the viruses of group B.

3. Redistilled ethyl ether, one hour at +4° C with agitation every ten minutes.

4. Combined action of Tween 80 and ether under the conditions prescribed by Muangay and Rott [24] according to Nosaka et al. [18] (virus suspension 1 vol., Tween 80 stock solution 1/20 (v/v) 1/10 vol., fifteen minutes, 28° C, subsequent extraction with one volume of ethyl ether, fifteen minutes, 28° C).

This treatment results in the dissociation of the resistant hemagglutinin from the infectious characteristics: the hemagglutinating titer remains unaffected or increases, as a function of the virus species and of the treatment. The results are given in Table 4.

Table 4. Effect of Phospholipase A, of Detergents and of Ether on the Hemagglutinating Titer

Virus	Preparation	Phospholipase A	Na Désoxycholate	Ether	Tween-Ether
Sindbis	Brain-acetone		—	97	—
	Culture	100	—	233	186
Chikungunya	Brain-acetone	—	100	110	100
West-Nile	Brain-acetone	50	100	100	—
Bunyamwera	Brain-acetone	100	80	140	110
	Culture	—	—	350	100

The results are given in percent of the hemagglutinating titer after treatment as compared to the original titer. Both titers are given in logarithmic units to the base 2. They are the average of a series of experiments.

c) The increase of the hemagglutinating titer, after treatment with ether or Tween-ether, must be interpreted as a function of the following facts obtained with the Sindbis and Bunyamwera viruses from cell cultures (the gel-filtrations of the Bunyamwera virus are, however, made from brain-acetone).

1. The control obtained by treatment of the noninfected cell culture medium is negative.

2. The titers calculated from various initial dilutions are consistent.

3. Treatment of a series of dilutions (geometric progression by 1/2) of the original virus -- diluted prior to treatment -- does not increase the apparent titer; the effect is on the virion itself (and not on the removal of a possible inhibitor or on the conditions of the reaction).

4. The treated preparations are inhibited by a specific antagonistic serum of the same titer as the original preparation (titer calculated for various antigen dilutions).

5 and 6. The optimum pH zone and the modes of action of various reagents (iodoacetamide, cysteine, urea) are identical for the original virus and for the treated preparations.

7. The treated preparations cannot be precipitated by protamine (final concentration used: 2.5 mg/ml).

8. The size of the treated particles is evaluated by filtration on a column of agarose granules. The results are assembled in Table 3, and examples shown in Figures 4 and 5. The action of ether (Bunyamwera) or of Tween-ether (Sindbis) results in a rupture of the virus particles and in the formation of a population of smaller particles, which probably are of a heterogenous size. This has also been shown by Mussgay and Rott [24] by specific methods, for Sindbis virus only.

This rupture of the virion explains the inactivation of the infectiveness under normal conditions and justifies the extraction of the infectious ribonucleic acids in a hypertonic medium [3, 33].

Discussion and Conclusions

1. The possibility of bonding and complex formation between lipids or lipoproteins and virions, shown elsewhere [26, 27], constitutes the major problem of the chromatographic study of the arboviruses in particular and of their purification in general. When such a complex is present, the bonds are broken by cold acetone; no satisfactory solution has been found in spite of the attempts at separation chromatography between a chloroform layer on water-repellent silicic acid and a mobile aqueous layer.

The existence of such complexes seems to be the logical explanation [30] of the heterogeneity noted during density gradient centrifuging of infectious brain suspensions. The slow moving peak is infectious, as is the fast peak [19, 31].

2. The rupture of the virions into sub-units, by ether treatment, is a general characteristic of lipoviruses which has already been studied extensively for myxoviruses [12, 21]. The results given here confirm the results of Cheng [9] and Mussgay and Rott [24]. The careful elimination of the lipoprotein inhibitors, especially those of sero-a origin, from the culture medium, makes it possible to obtain infectious and hemagglutinating particles. The infectious virion cannot be separated from the hemagglutinin either by the use of procedures based on the structure of the coat (ion exchange cellulose) or by procedures based on the size of the particles (agarose). The rupture of the particle, which results in its inactivation, is associated with a parallel, though heterogenous, decrease in the size of the hemagglutinins. Under favorable conditions these phenomena correspond to an increase in the number of hemagglutinins. It thus seems justified to conclude that the virion -- the infectious particle -- is the support of the hemagglutinin. The complement fixation properties have not been studied from this point of view. The results are identical for the investigated viruses, especially Sindbis (group A, size 42 mμ [6] or 60-70 mμ [24]) and Bunyamwera (group D, size 70 to 105 mμ [36]) and seem to indicate that these arboviruses have a similar architecture. They disagree with the findings of Kitaoka and Nishimura [20], who report that the major portion

of the hemagglutinins of the Japanese encephalitis virus (group B) sediment, after a two-hour-long centrifugation, at 40,000 t/m; while at the same time -- because of their 10 mμ size -- they can also be separated from the 50 mμ virions by density centrifugation. No satisfactory explanation of these contradictory facts is given. The soluble hemagglutinins described by Smith and Holt /36/ and by Gajdamovic and Doan /13/ have not been found and cannot be equated with the hemagglutinating units produced by the rupture of virions because they are not precipitated by protamine. The fractions, from brain suspensions treated with acetone, which diffuse across the agarose gel, have not been found in viruses obtained from tissue cultures in media devoid of inhibitor: they thus presumably are degradation products formed during the extraction with organic solvents.

3. Morphological studies /8, 23, 24, 17/ have shown that arborviruses consist of a coat and an inner, spherical core. The rupture of the coat by detergents and ether implies that the coat is so structured that the lipids constitute the tie between the protein structures /27/ which carry the hemagglutinating site. The morphological aspects have already been discussed by Mussgay and Rott /24/. These lipids come from a material preexisting in the host cell (Pfefferkorn and Hunter /29/).

The complexity of these hemagglutinating units, whether they are molecular associations or single molecules which carry specific sites, remains to be investigated. We have already reported on the distinct characteristics of the affinity for serum lipo-protein inhibitors and on the affinity for the receptor sites of the erythrocytes /26/. Symmetrically, one can conceive of a site for the fixation of antibodies separate from the hemagglutinating site and of an immunological inhibition based on steric hindrance or on an allosteric phenomenon. The particles produced by detergents or by ether, however, still fix specific antibodies. Furthermore, attempts to separate, with trypsin or with iodoacetamide, two hypothetical sites for the viruses of group A whose hemagglutinin resists the enzyme and the alkylating reagent, have failed. It is, however, important to clarify this point because the hemagglutinating units seem to preserve both an antigenic and an immunogenic activity (Mussgay and Rott /24/), this seems to indicate the existence of a cell receptor per site on the virus surface.

Summary

1. Chromatography on ion exchange cellulose permits the separation of the virus studied. No soluble hemagglutinin has been found.

2. Gel-filtration on agarose columns demonstrates a homogeneous behavior of infectious and hemagglutinating properties of the original preparations. Viral particles break when subjected to the action of ether and detergents; the break is associated with loss of infectious capacity only; hemagglutinating units obtained with this method preserve certain characteristics of the virions from which they are issued (immunologic specificity, inactivation, characters). The significance of these findings for the virion architecture is discussed.

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